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TECHNICAL NOTE

A micromethod to measure ammonia

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Although considerable quantities of ammonium are excreted in the urine, the current technology for studying nephron physiology demands the ability to perform assays on very small samples (micropuncture or microcatheterization samples) to determine the sites of ammonia addition to tubular fluid and the factors that regulate this process. Several techniques are available to measure ammonia, and the major ones were reviewed by Cunnaro and Weiner [1]. More specialized or laborious methods are required, however, to measure much smaller quantities of ammonia; that is, an antimony electrode [2]. We have recently developed a method to measure ammonium in nanoliter volumes of tubular fluid. The method to be reported is simple, reliable, reproducible, and more sensitive than standard fluorimetry [3, 4]. In principle, ammonium is quantitatively converted to ^{14}C- glutamate in the presence of an excess of ^{14}C-2-oxoglutarate (2-OG) and NADH when the enzyme glutamate dehydrogenase (GDH) is added. The reaction is stopped with hydrochloric acid. In very acidic solutions, 2-oxoglutaric acid is uncharged, whereas glutamic acid will have a net positive charge. Accordingly, the latter compound will be bound to Dowex-50 cationic exchange resin, whereas the 2-oxoglutaric acid will not. Hence, complete separation can be achieved. Measurement of the ^{14}C-glutamic acid will quantitatively reflect the amount of ammonia that was originally present.

Methods

Materials. Triethanolamine hydrochloride (TEA), NADH, 2-OG, and disodium adenosine diphosphate (ADP) were obtained from Boehringer-Mannheim, Montreal, Quebec. EDTA, disodium salt, GDH in glycerol buffer (ammonia-free), and Dowex-50 W, hydrogen ion form, 8% cross-linked, 50 to 100 mesh were obtained from Sigma Chemical Company, St. Louis, Missouri. Uniformly labeled ^{14}C-2-oxoglutaric acid in 0.1 N hydrochloric acid and uniformly labeled ^{14}C-glutamic acid were obtained from New England Nuclear, Lachine, Quebec. Citric acid, hydrochloric acid, and ammonium hydroxide were of Analar grade and obtained from British Drug Houses, Toronto, Canada.

Ammonia incorporation into radioactive glutamate. The buffer was prepared as follows: TEA (1.3 g) and EDTA (0.13 g) were dissolved in glass-distilled water. The pH was adjusted to 8.0 with 10 N potassium hydroxide, and the final volume was made up to 100 ml. This solution was divided into small portions and could be stored for months in the freezer. To prepare the assay mixture, we weighed 1.5 mg of 2-OG, 7.1 mg of NADH, and 0.5 mg ADP fresh daily and added them to 10 ml of buffer. Depending on the number of assays to be done, a portion of this assay mixture was selected; GDH (20 μl/ml) and uniformly labeled ^{14}C-2-OG (approximately 2 to 20 μCi/ml depending on the sensitivity required) were also added (that is, the lowest ammonia sample should have about 2000 cpm above the blank). The GDH and radioactive 2-OG were added just prior to the assay if nanomole sensitivity was required. If picomole sensitivity was required, the radioactive 2-OG was added 15 min after the GDH and just prior to the addition of the sample. The total volume was 50 μl for nanomole sensitivity and 1 μl or less for picomole sensitivity. Incubations were carried out at room temperature for 10-min and were terminated by the addition of 0.5 ml of 1 N hydrochloric acid. Samples were then frozen until analyses were done. No special precautions were used with respect to glasswashing or the production of an "ammonia-free" environment.

A minimum of four blanks and several standards (nanoliter volumes of 0.01 to 0.1 N, ammonium chloride) were also included with each experiment

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to avoid the requirement of quantitative recovery of radioactive glutamate. Nevertheless, the quantity of ammonia converted to ^{14}C-glutamate was virtually 100% in this reaction.

Separation of ^{14}C-2-oxoglutaric acid and ^{14}C-glutamic acid. Long-stem funnels were treated such that the bottom end had a constriction. A plug of glass wool was inserted down to this narrowing. A mini-column of Dowex-50 (0.5 × 5 cm) was equilibrated with 30 mM citric acid (pH, approximately 2.0). The total sample was then carefully added to the top of this column with a long-stem Pasteur pipette, and several small washings (citrate buffer, 30 mM; pH, approximately 2.0) of the tube were also applied to the column. Following this, the columns were washed with about 50 ml of the same citric acid solution (two funnels full), and all the effluents were discarded.

The columns were then washed with six 1-ml aliquots of 1 N ammonium hydroxide. The first 1-ml portion was generally discarded, as it contained little radioactivity. The ^{14}C-glutamic acid usually eluted in the 3rd or 4th ml. Each 1-ml was placed in 15 ml of scintillant (ACS, Beckman Company) and counted for ^{14}C-radioactivity. The usual recovery of ^{14}C-glutamate from the cation exchange column was 90 ± 4%.

Results

In a typical assay for nanomoles of ammonia, the mean value for the blank was 2296 ± 61 cpm (N = 4, mean ± SEM), whereas a 4-nmole standard had 10562 ± 327 cpm (N = 6). When the assay was designed to measure picomoles of ammonia (1 μl total volume), the blank was 682 ± 24 cpm (N = 4) and the 100-pmole standard had 3030 ± 62 cpm (N = 4). If GDH was omitted from the reaction mixture, virtually no radioactive glutamate was recovered. Representative standard curves are shown in Fig. 1, a and b.

When a standard quantity of ammonium was added to urine samples, its recovery was virtually complete (Table 1). In addition, the ammonium content in milliliter volumes of urine when measured by formol titration produced similar results to the assay of 10-nl portions of that urine when the method described in the present paper was used (106 ± 4%, N = 75).

Discussion

It is well known that the combination of radiochemical and enzymatic methods can increase the sensitivity of detection over the usual fluorimetric techniques [5]. One must, however, separate completely the small quantity of radioactive product from the large excess of radioactive substrate in this particular reaction. In the method described in this paper, we have used ion exchange resins for this purpose. One can readily separate the positively charged product glutamic acid from the uncharged

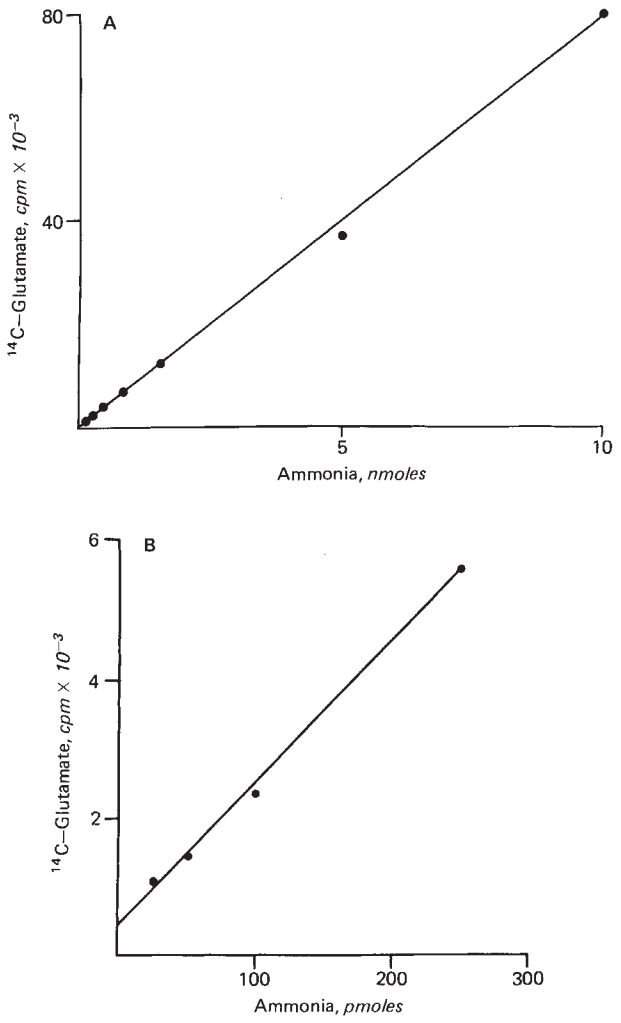

Fig. 1. **A** *Standard curve for assay of nanomoles of ammonia.* **B** *Standard curve for the assay of picomoles of ammonia.*

Table 1. Recovery of ammonium from urine samples

	Ammonium, *nmoles*			
Experiment	Urine	Standard	Urine and standard	Expected
1	1.35	0.71	2.05	2.06
2	2.90	0.71	3.63	3.61
3	0.40	4.00	4.30	4.40
4	4.80	5.00	9.80	9.90
5	4.60	5.00	9.60	9.60

2-oxoglutaric acid at a pH of 2 using small quantities of cation exchange resin. By extensive washing, virtually 100% of 2-oxoglutaric acid was removed before elution of ^{14}C-glutamic acid.

Another advantage of this method is convenience—nanoliter volumes are added directly to the assay medium when taken. A short time later, an excess of hydrochloric acid is added to arrest the reaction. Samples can then be stored in the freezer for processing at a later date. This method produced results for urine ammonium that were virtually identical to those done by conventional techniques.

Three possible pitfalls must be considered. Should one wish to assay ammonium in a solution that contains relatively large quantities of 2-OG, one would have to measure the resulting change in specific activity of 2-OG. This could be done by using an internal ammonium standard (adding a known quantity of ammonium to a portion of this fluid) or two different concentrations of nonradioactive 2-OG. Such high concentrations of 2-OG are, however, unlikely to occur in tubular fluid samples. Second, certain amino acids such as aspartate or alanine might react with 2-OG to produce glutamate if the appropriate transaminase were present. The commercial GDH preparation was less than 0.01% contamination with these transaminases, and these amino acids were not assayed by this technique. The tubular fluid samples are also unlikely to be contaminated with these transaminases. Hence, this problem with the assay should only become a problem in very unusual circumstances. Third, glutamate, if present in high concentrations relative to ammonium, can falsely elevate the value for ammonium because of isotopic exchange in the GDH reaction.

Summary. We have described a simple technique for the measurement of picomoles or nanomoles of ammonium. Samples can be easily processed on the day of an experiment. Analysis of ^{14}C-glutamate can be done at a later date by an accurate sensitive and reliable method. We can readily process 50 samples per day. The sensitivity can be increased by using smaller volumes of ammonia-free solutions, prereacting endogenous ammonia with nonradioactive 2-oxoglutarate, and increasing the specific activity of 2-oxoglutarate. The volumes must, however, be very low so as not to diminish the ammonium concentration and thereby limit glutamate formation.

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